UNIVERSITI PUTRA MALAYSIA

PURIFICATION OF CITRUS TRISTEZA VIRUS AND GENERATION OF MONOSPECIFIC POLYCLONAL ANTISERUM

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirement for the Degree of Master of Agricultural Science

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DEDICATION

to my wife Rini,
to my daughters and sons: Nuri, Zaldi, Nina, and Farhan,
you are really supporters, best friends, and assistants,
without your understandings, suggestions, patience, and endless praying,
this work could never have been completed.

to my parents, Moh. Usman Solichan and Zubaidah, (in memory)
to my parents-in law, A. Cholik Syukur and Minartiningsih,
and to my family members,
without your sacrifice, patience and endless praying,
many problems faced during this study
might not have been solved.

finally, but not least, to Allyu (in memory),
you were, and still are, a constant source of inspiration, spirit, and encouragement,
nothing is going to change my love for you.

it is appropriate that this work be dedicated to you all.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia
in fulfillment of the requirement for the degree of Master of Agricultural Science

PURIFICATION OF CITRUS TRISTEZA VIRUS AND GENERATION OF MONOSPECIFIC POLYCLONAL ANTISERUM

By

NURHADI

July 2002

Chairman : Associate Professor Kamaruzaman Sijam, Ph.D.

Faculty : Agriculture

Symptomatology, molecular weight of coat protein (CP) determination and purification procedure for citrus tristeza virus (CTV) antigen were studied to generate monospecific polyclonal antiserum. Results of survey indicated that CTV was found to attack citrus varieties such as *C. grandis, C. aurantifolia, C. reticulata, C. hystrix, C. nobilis* and *C. sinensis* with variable symptoms and degrees of severity. Virus complex varied among citrus varieties and cultivars, and there were four important sub isolates found, namely mandarin stem pitting, orange stem pitting, pomelo small fruit, and pomelo mild isolate.

In partial purification step, antigen was concentrated by polyethylene glycol (PEG) precipitation followed by low speed centrifugation. Semi purified

antigen was then finally purified by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). A major protein band containing CP was excised and eluted using elution buffer containing 0.25 M Tris-HCl pH 6.8 + 0.1 % SDS. With a starting material consisting of 50-gram bark tissue of semi dormant flush, 750 µg of eluted CP as monospecific antigen was obtained. The use of PEG and NaCl for virus precipitation combined with low speed centrifugation in semi purification step, then followed by electrophoresed of semi purified virus preparation in final purification step effectively minimized virus losses during purification processes and minimized possible contamination of plant components in the final immunogen.

Monospecific polyclonal antiserum against citrus tristeza virus was generated using the viral CP. Upon SDS-PAGE of local CTV isolate designated as UPM/T002, two protein bands specific for CTV with molecular weight of 25 kDa and 33 kDa were obtained. The major band with molecular weight of 25 kDa that reacted strongly with commercial polyclonal antibody in double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA), was used as the antigen for injection into female White Leghorn chicken.

Chicken immunoglobulin recovered from immature egg reacted strongly with semi purified CTV up to 1:4096 dilutions in microprecipitine test. In DAS-ELISA, using sap of infected plant, the sensitivity of coating

immunoglobulin and enzyme conjugate were 1:1,000 and 1:500 dilutions respectively. Immunoglobulin reacted specifically with CTV isolate and gave no background reaction to healthy plant sap. The simplicity of the procedure makes it economically acceptable and technically adoptable because the antigen can be prepared with limited chemical and equipment. This study is the first reporting antiserum production from CTV-CP using local CTV isolate from Peninsular Malaysia.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains Pertanian

PENULENAN CITRUS TRISTEZA VIRUS DAN PENJANAAN POLIKLONAL ANTISERUM MONOSPESIFIK

Oleh

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Pengerusi : Profesor Madya Kamaruzaman Sijam Ph.D.

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Pengenalpastian simptom, penentuan berat molekul kod protein dan kaedah penulenan untuk antigen citrus tristeza virus (CTV) telah dikaji untuk menghasilkan antiserum poliklonal monospesifik menggunakan isolat CTV setempat. Hasil survei menunjukkan bahwa CTV dijumpai menyerang varieti limau seperti *C. grandis, C. aurantifolia, C. reticulata, C. hystrix, C. nobilis* dan *C. sinensis* dengan simptom berbeza dan berbagai tingkat keparahan. Kompleks virus berbeza diantara varieti dan kultivar dan terdapat empat subisolat penting iaitu 'mandarin stem pitting', 'orange stem pitting', 'pomelo small fruit' dan 'pomelo mild isolat'.

Pada peringkat awal penulenan, antigen dipekatkan dengan menggunakan kaedah presipitasi polyethylene glycol (PEG) diikuti dengan pengemparan

berkuasa rendah. Antigen semi tulen selanjutnya ditulenkan dengan kaedah dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Jalur utama protein yang mengandungi CP dipotong dan ditulen menggunakan 'elution buffer' yang mengandungi 0.25 M Tris-HCl pH 6.8 + 0.1 % SDS. Bahan awal seberat 50 g tisu kulit batang yang diperolehi daripada pucuk semi dorman berjaya menghasilkan 750 ug CP CTV sebagai antigen monospesifik. Penggunaan PEG dan NaCl untuk presipitasi virus yang dipadukan dengan pengemparan berkuasa rendah pada step semi penulenan, yang diikuti dengan elektroforesis daripada virus semi tulen pada step penulenan akhir, sangat berkesan untuk meminimunkan kehilangan virus semasa proses penulenan dan meminimumkan kehadiran bahan-bahan kontaminasi daripada komponen tumbuhan pada hasil akhir penulenan.

Antiserum poliklonal yang spesifik terhadap CTV telah berjaya dihasil menggunakan viral CP. Dengan menggunakan isolat CTV setempat UPM/T002, melalui analisis SDS-PAGE, dua jaluran yang mempunyai berat molekul 25-kDa dan 33-kDa dan spesifik terhadap CTV telah dihasilkan. Jaluran tebal yang berat molekulnya 25-kDa dan bertindakbalas kuat dengan antiserum komersial ketika diuji dengan double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA), digunakan sebagai antigen untuk tujuan imunisasi pada ayam telur jenis White Leghorn.

Immunoglobulin yang ditulenkan daripada telur belum sempurna berinteraksi kuat dengan CTV semi tulen sehingga tahap pencairan 1:4096 semasa ujian mikropresipitin. Pada ujian DAS-ELISA, dengan menggunakan sap daripada tanaman berpenyakit, sensitifiti 'coating immunoglobulin' adalah 1:1,000 pencairan, manakala enzim konjugat pula pada pencairan 1:500. Immunoglobulin bertindakbalas spesifik terhadap isolat CTV dan tidak menunjukkan reaksi tidak spesifik terhadap sampel sap sihat. Kesederhanan kaedah penulenan dalam kajian ini menjadikannya dapat diterima dari segi ekonomi dan teknikal kerana antigennya dapat disediakan pada keadaan peralatan dan bahan kimia minimum. Kajian ini merupakan kajian pertama yang melaporkan penghasilan antiserum daripada telur ayam yang belum sempurna, yang dihasilkan daripada viral CP menggunakan isolat CTV setempat dari Semenanjung Malaysia.

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I certify that an Examination Committee met on 23rd July 2002 to conduct the final examination of Nurhadi on his Master of Agricultural Science thesis entitled "Purification of Citrus Tristeza Virus and Generation of Monospecific Polyclonal Antiserum" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfillment of the requirement for the degree of Master of Agricultural Science. The members of the Supervisory Committee are as follows:

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I hereby declare that the thesis is based on my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

NURHADI BIN USMAN SOLICHAN

Date: 22 August 2002

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APS	Ammonium peroxydisulphate
BIS	N. N'–Methylenbisacrylamide
BPB	Bromophenol blue
BSA	Bovine serum albumin
CTV	Citrus tristeza virus
DAS-ELISA	Double-antibody sandwich enzyme-linked immunosorbent assay
DE-52	Diethylaminoethyl cellulose
DNA	Deoxyribonucleic acid
hr	Hour
kDa	Kilodalton
µl	Microliter
M	Molar
mg	Milligram
min	Minute
nm	Nanometer
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
TEMED	N, N ,N, N, -Tetramethylethylendiamine
Tris-HCl	Tris-(hydroxymethyl)–aminomethane hydrochloric acid

Alkaline phosphatase	An enzyme which hydrolyses certain phosphate-containing compounds under alkaline conditions; commonly obtained from calf intestine mucosa.
Antibody	A protein formed in blood serum in response to stimulation by an antigen. Antibodies are specific for their respective antigens, and antigens and antibodies are mutually attracted.
Antigen	A substance which, when introduced into the biological environment of a vertebrate animal, leads to the formation of antibodies directed specifically against it. All immunogens are antigenic, but not all antigens are immunogenic.
Antigenic Determinant	The small site on the antigen to which antibody is specifically able to become attached as determined by structural complementarily between antibody and antigen molecules.
Antiserum	Serum from any animal containing antibodies to a specified antigen.
Conjugate	The product of joining two or more dissimilar molecules by covalent bonds. In immunological contexts, one is usually a protein and the other either a hapten or a label such as fluorescein, ferritin, or enzyme.
Dialysis	A procedure using a membrane to separate various components in solution in accordance with their ability to pass through the membrane
Epitope	An antigenic determinant of defined structure, e.g., an identified oligosaccharide, or a chemical hapten.
Freund's Adjuvant.	A mixture of mineral oil and lanolin that enhances immune responses when emulsified with antigen for immunization. Freund's complete adjuvant includes killed mycobacteria; Freund's incomplete does not.

Immunization	Administration of antigen to an animal so as to produce an immune response to that antigen.
Immunogen	A substance that elicits an immune response when introduced into the tissues of an animal. To stimulate a response, immunogens must normally be foreign to the animal to which they are administered, of a molecular weight greater than 1,000, and of protein or polysaccharide nature.
Immunoglobulin (Ig).	Serum globular glycoprotein. There are five classes of immunoglobulin, IgA, IgD, IgE, IgG, and IgM. IgG is the major immunoglobulin class in the serum of man and in most species from amphibians upwards.
Polyclonal antibody	An heterologous antibody population derived from many clones.

CHAPTER 1

INTRODUCTION

1.1 Economic Importance of Citrus

Citrus is one of the most important fruit crops grown in all continents of the world. It contributes to the nourishment and refreshment of the people. Citrus products and by-products provide the basis for local agricultural industries, generate employment, raise income and, in many cases, constitute an important source of foreign revenue to developing and developed countries. Citrus production developed significantly during the last decade. From 1985 to 1995 world production increased at approximately 66 % from 47.8 Mt to 79.3 Mt. Oranges occupy the major part of the market, followed successively by mandarins, lemons, pomelos and other types (Table 1.1).

Table 1.1: Trend of citrus production from 1985 to 1995.

Citrus Types	Production campaigns			
	1985-1986		1995-1996	
	MT	%	MT	%
Oranges	31.0	65.7	58.4	73.0
Mandarins	7.9	16.7	8.8	11.0
Lemons	4.3	9.1	6.9	8.0
Pomelos and Grapes	4.0	8.5	5.2	8.0
Total	47.8	100.0	79.3	100.0

Source: FAO (1996)
MT: Metric tones

In Malaysia citrus has been cultivated since late 19th century and it was reported to be an economically viable plantation crop in the 1920's (Bunting, 1929). In the 1960's, citrus cultivation was popular among small land holders but diseases and pests caused a decline resulting in many growers abandoning their citrus plantings. Thus, citrus production decreased from 3670 ha in 1970 to 2487 ha in 1985. In 1995, the area cultivated throughout Peninsular Malaysia surpassed that of 1975 with 4291 ha planted with citrus. Out of these 1820 ha were planted with pomelo, 1295 ha with mandarins and the remaining 1176 ha consisting mainly of varieties of indigenous types such as sweet orange, limes and citrons (Table 1.2). With the increasing population, it is projected that Malaysia would need at least 12,500 ha of citrus to satisfy local demand (Ko, 1996).

Among commercial citrus varieties grown, oranges (*Citrus sinensis* L. Osb.), tangerines (*C. sinensis x C. reticulata.*), mandarins (*C. reticulata* Blanco), lemons (*C. limon L.* Burmf.), limes (*C. aurantifolia* Christm. Swing.), pomelos (*C. grandis* L. OSB.), and grapefruits (*C. paradisi* Macf.) are widely grown. Even though grapefruit was probably among the earliest citrus type introduced by the Department of Agriculture (Hawson, 1958), pomelos and especially mandarins were the most popular variety cultivated commercially. Limau Langkat *(Citrus suhujensis* Hort. ex Tan), known locally as the popular loose peel citrus, can produce yields of up to 35 tones/ha/yr. yielding a gross income of RM 52,000. The recent introduction